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Patterning Pluripotent Stem Cells at a Single Cell Level

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Abstract

Studies of cell-extracellular matrix (ECM) interactions at a single cell level have drawn interest from scientists around the world. Subcellular ECM micropatterning techniques allow researchers to control cell shape, migration, and spindle orientation during mitosis potentially influencing the stem cell fate. Generally these studies have been limited to somatic cells rather than human pluripotent stem cells (hPSCs) which are capable of enormous differentiation potential. hPSCs require a defined ECM for attachment and express characteristic integrins mediating cell-substrate interactions. hPSCs also rely on cell-cell contacts for survival and to maintain self-renewal properties, but these circumstances also significantly limit hPSC observation at a single cell level. In addition, currently available methods for ECM micropatterning generally require a facility with trained personnel and intricate equipment to produce protein micropatterns. To overcome this problem, we have developed a protocol for vitronectin micropatterning using simple UV/ozone modification of polystyrene. Single hPSCs were able to attach and form characteristic stress fibers and focal adhesions similar to somatic cell types which demonstrate hPSC responsiveness to extracellular adhesive cues. Micropatterned hPSCs were able to be cultured for up to 48 hours while maintaining expression of pluripotency-associated transcription factor OCT4. Although further studies are necessary, the results of our investigation will potentially have a large impact on cell regenerative medicine and tissue engineering.

Keywords

Pluripotent Stem Cells; Micropatterning; Extracellular Matrix; Stress Fibers; Polystyrene; UV/Ozone; Vitronectin

1. INTRODUCTION

Methods such as dip pen nanolithography, microcontact printing, and direct photo-patterning using UV light or laser to deposit adhesive proteins are attractive options for cell biologists studying molecular processes and cell-material interactions at a single cell level.¹ Features designed by these approaches can be fabricated down to nanometer scale and are generally produced utilizing self-assembling monolayers as a method for protein adsorption. Random

studies of UV/ozone surface functionalization of polymers such as polyethylene and polyvinyl chloride (PVC) have also been reported.^{2,3} Polystyrene (PS) is traditionally used for cell culture applications where the PS is plasma-treated to render it more hydrophilic and promote cell attachment.^{4,5} A few research groups have also developed methods to apply direct UV/ozone for micropatterning of PS in cellular studies.^{6–8} These methods are generally more cost effective and can be easily implemented in biology labs.

Micropatterning has been used to study internal cell organization, cell division, migration, or simply to control cell outgrowth.^{1,8} Tissue morphogenesis and homeostasis in living organisms are highly dependent on the local microenvironment where cell–cell and cell–ECM interactions play crucial roles.⁹ Adhesive boundaries are able to dictate cell positioning and influence cell behavior. External constraints can affect cell polarization within a tissue and control the cell division axis, thus determining a cell fate.^{10–12} Disbalance of tissue homeostasis and disruption of cell polarity can also pose adverse consequences potentially leading to cancer.¹³ Engineering cell microenvironments at a microscale *in vitro* allows researchers to study single cells and multicellular groups under close to native conditions.¹ While established cell lines and adult stem cells such as human mesenchymal stem cells (hMSCs) are widely used in these studies, hPSC micropatterning remains under development. hPSCs are capable of differentiation into nearly all types of human cells and are of great interest for cell biologists as well as clinicians.^{14,15} The opportunity to investigate internal organization, cellular processes, and cell–cell interactions in hPSCs at a single cell level opens new perspectives for regenerative medicine and tissue engineering.

hPSCs have been reported to be highly sensitive to external microenvironmental cues and necessitate specific cell culture conditions. hPSCs such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) grow in colonies and require a supporting feeder layer or a specific ECM to support their undifferentiated state.^{14–16} Disruption of cell–cell contacts usually affects actin-myosin cytoskeleton structure and induces changes in expression of adhesion molecules along with pluripotency-associated transcription factors such as OCT4, SOX2, NANOG, and MYC.¹⁷ Single-cell dissociation of hPSCs has been reported to induce cell apoptosis.^{18,19} However recent studies have demonstrated the possibility to grow cells in a monolayer culture or in colonies with single-cell passaging using ROCK inhibitor to eliminate apoptosis during cell dissociation. This suggests the possibility of hPSCs being plated on micropatterns for single cell studies.^{8,20,21}

Under feeder-free conditions hPSCs have been traditionally cultured on Matrigel, which forms a gel after the polymerization of a few constitutive proteins and heparan sulfate. Due to this protein polymerization layer Matrigel is generally an unsuitable source for micropatterning.^{22,23} Studies have shown that hPSCs express integrins mediating cell binding to vitronectin which is able to replace Matrigel and support undifferentiated hPSC growth in culture.^{16,24} With this in mind vitronectin provides an ideal protein for the micropatterning of hPSCs at the single cell level. Methods for high-resolution vitronectin micropatterning have yet to be developed and here we report a simple, affordable, and fast protocol to fabricate vitronectin micropatterns with feature resolution to 1 μm . We also demonstrate that single hPSCs are able to attach to micropatterns and reorganize cell shape

and cytoskeleton to pattern, and maintain expression of pluripotency-associated marker OCT4 for up to 48 hours in culture. These results provide the basis for future hPSC studies at subcellular levels and can be successfully used for studies of both hPSCs as well as diverse differentiated cell types.

2. MATERIALS AND METHODS

2.1. Mask Design and UV/Ozone Micropatterning of Polystyrene

Design and sketching of micropatterns was performed in AutoCad Design Suite. Super high resolution chrome quartz photomasks (positive and negative) sized 10 cm × 10 cm were ordered from J. D. Photo-Tools, UK. Clear PS sheets 0.3 mm thick were ordered from Plastruct (#SSM-101). PS sheets were cut to approximately 2 cm×2 cm square coverslips to fit wells of six-well cell culture plates. Before UV/ozone patterning PS coverslips were disinfected for one hour in freshly prepared 70% ethanol (Decon Labs) and washed once in sterile distilled water.

A UV/ozone ProCleaner (BioForce Nanosciences) was used for direct UV micropatterning. The UV lamp was preheated for 15–30 minutes before patterning. Quartz photomask was washed with isopropanol, dried under air flow, and then hydrophylized in UV/ozone ProCleaner with feature side up for about 10–15 minutes. PS coverslips were deposited on desired patterns with a 4 μ l drop of water to ensure close contact with the chrome side of the mask. PS coverslips/quartz photomask sandwich was exposed for 2.5 minutes to UV light from the distance of approximately 3 cm from UV lamp. Coverslips were then removed from the mask by adding water around coverslips and allowing them to be lifted from the surface minimizing photomask damage. Samples were used for protein coating and cell plating immediately or analyzed by XPS within 1–2 hours.

2.2. XPS and Data Analysis

XPS measurements were conducted using a Kratos AXIS Ultra DLD XPS system equipped with a monochromatic Al Ka source. The binding energy was calibrated using an Ag foil with Ag3d_{5/2} set at 368.21 ± 0.025 eV for the monochromatic Al X-ray source. The monochromatic Al Ka source was operated at 15 keV and 120 W. The pass energy was fixed at 80 eV for the detailed scans. A charge neutralizer was used to compensate for the surface charge. The binding energy scale for C1s was set at 285 eV. Elemental surface compositions (atomic%) were calculated based on C1s and O1s detailed scan spectra.

Plastic used in these studies:

1. bacterial grade cell culture PS, 10 cm Petri dishes (Greiner, Cat. No. 663 161 or 664 161), tissue culture treated PS, 6-well plates (BD, Cat. No. 353046);
2. PVC 22 × 22 mm coverslips (Electron Microscopy Sciences, Cat. No.72261-22);
3. clear PS sheets 0.3 mm thick Plastruct (#SSM-101).

2.3. Cell Culture

hESC line H9 was purchased from WiCell (Wisconsin)¹⁴ and the two bone marrow-derived hiPSC lines BM1M and BM9 were kindly provided by Dr. Slukvin (University of Wisconsin-Madison).¹⁵ hESC HES3 line was gratefully provided by Dr. Elliott (Monash University).²⁵ All cell lines were grown on mouse embryonic fibroblast (MEF) (CF-1, Millipore, #PMEF-CFL) feeder layer in DMEM/F12 (Invitrogen, #11330-057) with 20% Knock-Out SR (Invitrogen, #10828-028), 0.1 mM MEM Non-Essential Amino Acids (Invitrogen, #11140-050), 3.5 mM L-Glutamine (final concentration) (Invitrogen, #25030-081), 100 μ M 2-Mercaptoethanol (Sigma, #M7522) and supplemented with 10 ng/ml human recombinant bFGF (hrbFGF) (PeproTech, #100-18B) (also see WiCell Protocols). Medium was changed daily and cells were passaged on the fifth day of culture with collagenase type IV (Invitrogen, #17104-019).

MEFs were grown in DMEM (Invitrogen, #11995-073) supplemented with 10% FBS (Invitrogen, #10438-026, or Atlas Biologicals, #F-0500-A) and 100 U/100 μ g/ml of penicillin/streptomycin (Invitrogen, 15140-122).

hPSC monolayer culture with single cell passaging was established as described.²⁰ In brief, hPSCs were converted from feeder-dependent culture to feeder-free conditions and were grown as colonies for 2–3 passages on growth factor-reduced Geltrex-coated (1:400) (Invitrogen, Cat. No. 12760-021) 6-well plates (BD, Cat. No. 353046) in MEF-conditioned medium supplemented with 10 ng/ml hrbFGF. Cells were passaged mechanically on each fifth day. Conditioned medium was prepared as described:²⁰ MEFs were mitotically inactivated with 10 μ g/ml Mitomycin C (Sigma, #M4287) for 2.5 hours and then plated onto gelatinized flasks (Gelatin, Sigma, #G-1890) at a density of 60,000 cells/cm². The following day cells were washed with PBS (Sigma, #P3813) and medium was changed to hESC medium (without hrbFGF) in a quantity of 0.5 ml/cm² and collected every 24 hours for 7 days. Before cell feeding conditioned medium was filtered and supplemented with 10 ng/ml hrbFGF. On the next passaging hPSC colonies were treated with TrypLE Select (Invitrogen, #12563-029) for 1 minute and gently dissociated into single cells and plated to new wells at an approximate density of 80–100,000 cells/cm². Cells were grown under the same culture conditions and passaged as single cells upon reaching confluence. After stabilization of the cell culture cells were routinely passaged as single cells on each fourth day with a seeding density of 50,000 cells/cm². In the second round of experiments ROCK inhibitor Y-27632 (Calbiochem, #688000) was added in hPSC culture medium postseeding at a concentration of 10 μ M and kept overnight to prevent cell apoptosis and chromosome changes during prolonged culture. Cells were plated at density 20,000 cells/cm².

Bone marrow-derived hMSCs were obtained from Lonza (PT-2501) and cultured in hMSC basal growth media (PT-3001 MSCGM BulletKit, Lonza).

2.4. Protein and Cell Deposition on Micropatterned Polystyrene

For hydrophobic surface protein coating UV/ozone-patterned coverslips were incubated with 1 μ g/ml of human recombinant vitronectin (Sigma, #SRP3186) in DMEM/F12 (Invitrogen, #11330-057) for 30 minutes at room temperature (RT) and further washed three times with

DMEM/F12 and cells were deposited. For hydrophilic patterns polystyrene coverslips were pretreated with 0.1% solution of BSA (Sigma, #A2153) in PBS (Invitrogen, #14040-141) for 15–20 minutes followed by incubation with vitronectin as described previously. To visualize micropatterns, 1.5 $\mu\text{g}/\text{ml}$ of human fibrinogen Alexa Fluor 488-conjugate (Invitrogen, #F-13191) was added into DMEM/F12 with 1 $\mu\text{g}/\text{ml}$ of human recombinant vitronectin and further incubated and washed as described previously.

2.5. Karyotyping

Chromosome spreads were prepared by traditional G-banding technique and analyzed by KaryoLogic, Inc.

2.6. Immunocytochemistry

For immunostaining cells were washed with PBS (Sigma, #P3813), fixed with 10% formalin (Sigma, #HT50-1-1) for 20 minutes at RT, and permeabilized with 0.1% Triton-X 100 (Sigma, #T8787). To prevent non-specific antibody binding cells were incubated for 30 minutes at RT in blocking solution (PBS + 4% goat serum (Sigma, G9023)). Cells were then incubated with primary antibodies in blocking solution for 1 hour at RT, washed with rinse buffer (20 mM Tris-HCl + 0.05% Tween-20 (Sigma, #T5912, #P9416)), and incubated with secondary fluorochrome-conjugated antibodies. In some trials cells were further stained with Rhodamine-Phalloidin (Invitrogen, #R415) in PBS for 1 hour at RT and washed with rinse buffer. Antibodies used were: primary-OCT4 (Santa Cruz, sc-5279), SOX2 (Stemgent, 09-0024), Vinculin (Sigma, V9264), Vimentin (Santa Cruz, #sc-6260), secondary-IgG AF488 (Invitrogen, A31620), IgG AF594 (Invitrogen, A31624). Cells on glass coverslips (Electron Microscopy Sciences, #72230-01), PVC, or PS samples were mounted on glass slides (VWR, #16005-106) with DAPI containing mounting solution (Sigma, #F6057). Samples were analyzed and images taken using upright Nikon Eclipse 80i or inverted Nikon Eclipse Ti fluorescent microscopes with NIS-Elements imaging software.

3. RESULTS AND DISCUSSION

3.1. Mask Design and Selection of the Micropatterning Method

Mask and pattern design were created using AutoCAD Design Suite. Two identical but inverted (positive and negative, to produce either hydrophobic or hydrophilic single cell patterns) photomasks were used for studies as shown in Figure 1(A). Based on our preliminary data on single hPSCs on Cytoo chips (www.cytoo.com) the majority of cells attached to medium (1100 μm^2) and large (1600 μm^2) sized fibronectin patterns (data not shown). Thus, two sizes of patterns (disk, crossbow, T, H, Y) were considered for further applications: #1 1256 μm^2 (disk $d = 40/\mu\text{m}$) and #2 1962.5 μm^2 (disk $d = 50/\mu\text{m}$). Pitch between micropatterns was 100 μm with pattern line width 6.6 μm for #1 and 8.3 μm for #2. Single cell patterns were organized in six blocks (25 $\times$ 12) within 14 mm diameter circles to fit a well of 24-well plate. The total number of patterns per chip was 1800 (300/block) (Fig. 1(A)), which proved sufficient to provide statistically satisfactory analysis with 50–100 cells analyzed.⁷

Methods such as photolithography and laser/electron beam etching technique among others require complex equipment and trained personnel. Thus, for daily experiments in a biological laboratory traditional microcontact printing or UV-based chemistry would be the most facile.⁷ Microcontact printing consisting of mold preparation, PDMS stamping, and further protein patterning on self-assembled monolayers is still an expensive, time consuming, and complicated approach requiring corresponding skills. Glass and other materials are also difficult to resize without the appropriate tools but recent advances in substrate surface chemical modification based on direct UV light exposure offer a fast, simple, and inexpensive method for ECM micropatterning. A number of publications described UV-based patterning of different polymeric substrates such as polyethylene, polycarbonate, polymethylmethacrylate, and PS which are easy to manipulate and inexpensive.^{2,26} Moreover, this method was successfully employed for clonal growth of hiPSCs on UV-modified PS and polypropylene surfaces.⁸

Direct UV patterning methods consist of the treatment of a polymer surface with UV/ozone which modifies surface chemistry by adding oxygen to its structure, thus hydrophilizing a substrate and improving cell attachment and ECM protein deposition. Oxygen can also be added through plasma treatment or ion beam irradiation. For example successful patterning of cells on PVC was shown through surface functionalization by ion beam irradiation techniques.³ PS is routinely used for cell culture and is usually oxidized by plasma treatment.^{4,5}

3.2. Polymer Substrate Characterization

PVC is a widely used polymer in clinical settings for a variety of medical applications. Commercially available PVC coverslips (Electron Microscopy Sciences) are not autofluorescent and are often used for cell analysis in medical applications. However, micropatterning of PVC coverslips appeared unsuccessful in our trials. XPS analysis of UV/ozone-treated PVC surface revealed that it is exceedingly difficult to modify in comparison with cell culture PS (Table I) and it demonstrates a high rate of background fluorescence after UV/ozone exposure. In addition we revealed that PVC initially contains up to 9.35% bound oxygen as seen in Table I. In a previously published study by Mitchell et al. it was shown that the washing of oxidized PS with HPLC grade water for one hour resulted in partial removal of bound oxygen from polymer surfaces (up to 8%).⁶ Interestingly, soaking PVC and PS samples in 70% ethanol also decreased the oxidation level of plastic (Table I).

Untreated cell culture PS is optimal for chemical modification and has been efficiently used for micropatterning and growth of hPSCs from single cells, however resolution with the custom-made stainless-steel mask was only 30 μm which is not suitable for subcellular patterning.⁸ Furthermore, commercially available quartz chrome masks that allow resolution down to 1 μm cannot be used with commercially available cell culture plastic due to technical reasons including flexibility issues and the difficulty to resize thick PS. Thus, we decided to test 0.3 mm thin clear Plastruc PS sheets for UV/ozone micropatterning applications. This polymer was analyzed by XPS on surface oxygen binding capacity with different UV/ozone exposure doses and under different conditions (Table I).

Similar to PVC, Plastruct PS demonstrated low levels of oxygen (2.69%) initially present (Table I). Based on our previous data with ethanol treatment of PVC coverslips the same procedure minimized surface-bound oxygen of Plastruct PS up to 1.11% (Table I). Saha et al. have reported 2.5 minutes of UV/ozone exposure to unmodified cell culture PS as optimal for hPSC culture, while lower doses decreased cell attachment and higher doses generated ions negatively affecting cell growth.⁸ Mitchell et al. have shown that optimal oxygen concentrations on PS surfaces for 3T3-L1 and CHO cell growth were approximately 5–25% (< 100 seconds of UV/ozone treatment in UV/ozone cleaner (Jelight Company Inc.)).⁶ The authors explain this by changes in ECM and medium component interactions with the chemically modified PS. Therefore we analyzed oxidation levels of cell culture PS including unmodified PS, unmodified PS exposed for 2.5 minutes to UV/ozone and tissue culture treated PS (Table I). We further compared UV/ozone treatment parameters of Plastruct PS to that of cell culture PS optimized for hPSC culture, i.e., 2.5 minutes of UV/ozone treatment of unmodified PS (Table I).⁸ Results for ethanol-treated Plastruct PS samples appeared similar to cell culture PS (oxygen 18.16% vs. 19.07%) as seen in Table I, however it appeared that the clear quartz mask significantly decreased the level of bound oxygen after 2.5 minutes of UV/ozone exposure (oxygen 3.67% vs. 18.16%). The increase in exposure up to 7.5 minutes gave only slight shifts in the level of oxidation (oxygen 4.27%) (Table I).

3.3. ECM Protein and Cell Deposition on UV/Ozone-Micropatterned PS Coverslips

Unlike somatic cells hPSCs require a specific ECM for their undifferentiated growth. Matrigel (BD) or Geltrex (Invitrogen) have been successfully used for feeder-free culture of hPSCs. Matrigel consists of a few ECM proteins including laminin, collagen IV, entactin/nidogen, and heparan sulfate proteoglycan which polymerize above a temperature of 10 °C and form a gel.^{22,23} Studies have shown that hPSCs express the laminin+entactin receptor $\alpha 6\beta 1$, vitronectin receptor $\alpha V\beta 5$, and fibronectin receptor $\alpha 5\beta 1$ thus allowing their attachment to Geltrex or Matrigel as well as to vitronectin and fibronectin.^{16,24} However only vitronectin was shown as capable of supporting hPSC self-renewal and able to replace Matrigel in cell culture.^{16,24} Based on these findings we have chosen vitronectin as the ECM protein for micropatterns.

To visualize micropatterns, human fluorescently-labeled fibrinogen Alexa Fluor 488 was co-incubated with human recombinant vitronectin (Fig. 1(B)). At the low UV/ozone exposure dose (2.5 minutes) fibrinogen tended to attach to hydrophobic (unmodified) areas of PS samples (Fig. 1(B)). No cell attachment to hydrophobic single cell patterns was observed for hPSCs, MEFs, or hMSCs although some instances of non-specific cell attachment were observed. We hypothesized that under these conditions protein binding to chemically patterned PS samples might compete between hydrophobic and hydrophilic surfaces in the benefit of hydrophobic surface.

A previously published study by Welle et al. has shown that immobilization of laminin on high resolution micropatterns produced by UV modification of PS can be eliminated on non-treated (hydrophobic) surfaces and enhanced on treated (hydrophilic) surfaces by pretreatment with BSA/Pluronic F-68 followed by laminin/Pluronic F-68.²⁷ Thus BSA

pretreatment of UV patterned Plastruct PS substrates followed by vitronectin binding allowed us to decrease non-specific cell attachment to non-treated hydrophobic surfaces and achieve attachment and characteristic spreading of MEFs and hMSCs on hydrophilic micropatterns (Fig. 2). When attached to micropatterns cells formed typical stress fibers (Fig. 2) and focal adhesions (Fig. 2(F)). Interestingly, BSA prevented fluorescently labeled fibrinogen from binding to the patterned surface (data not shown).

To investigate how higher doses of UV/ozone exposure affect cell attachment to single cell patterns we UV/ozone treated Plastruct PS samples for 2.5 and 7.5 minutes. PS coverslips were pretreated with BSA and vitronectin as described previously and MEF cells were seeded on plastic at a density of 100,000 cells/chip. The cases of 2.5 and 7.5 minutes of UV/ozone treatment ultimately did not give significant variance in cell attachment (0.34 and 0.44% correspondingly, or 19% and 24% of patterns were occupied) and in the following experiments we applied 2.5 minutes for the PS patterning.

3.4. Micropatterning of Single hPSCs

Our previous data with Cytoo chips have shown that single hPSCs can be plated on micropatterns directly from enzymatically treated and resuspended colonies grown under feeder-free conditions or from a monolayer culture which includes single-cell passaging (data not shown). Recent studies have demonstrated that hPSCs can be grown as a monolayer culture for prolonged period of time without acquiring karyotypic abnormalities.^{8,21} Monolayer culture allows for the expansion of hPSCs as a homogeneous population in contrast to cells grown in colonies.^{8,20,21} Monolayer culture with single cell passaging is also more suitable for single cell studies, however our attempts to grow hESCs and hiPSCs for a period of over 10 to 20 passages with single cell passaging and in the absence of supportive feeder cells have resulted in the commonly reported extra chromosomes 12 and 20 in hESCs (H9) (Fig. 3(E)) and chromosome 12 in hiPSCs (BM9) (Fig. 4(C)) in each cell analyzed (5 metaphases total).²⁸ Similarly, Saha et al. have shown that monolayer culture in mTeSR1 medium with single-cell passaging resulted in chromosomal instability but the addition of ROCK inhibitor after cell dissociation has preserved normal hPSC karyotype.⁸ Thus we reestablished a monolayer cell culture from hESCs and began using ROCK inhibitor postseeding and found after 10 passages under these conditions about 50% of the cells demonstrated normal karyotype. However in contrast to Saha et al. the other 50% of hESCs revealed various missing and extra chromosomes with aneuploidy of chromosomes 14 and 20 being the most common (8 metaphases analyzed) (Fig. 3(F)). Thus, cell culture medium (mTeSR1 versus MEF-conditioned medium), higher surface oxygen concentration compared to standard tissue culture treated PS (Table I), or possible genetic background of hPSC lines might have an effect on chromosomal stability. In addition, our results suggest that the time of ROCK inhibitor treatment (overnight) also may be potentially damaging leading to even worse outcomes. Further studies are needed to optimize these single cell culture conditions, yet hPSCs from a monolayer culture could be used for single cell studies at lower passage numbers such as 2–4.⁸

We further studied hPSC attachment and spreading on vitronectin-coated single cell micropatterns. Single hPSCs from a monolayer culture (hiPSC BM9 and hESC H9 lines) or

from colonies (hESC HES3 line) were plated on patterns at a density of 250,000–500,000 cells/chip, allowed to spread and form stress fibers for 3 to 4 hours, and fixed for further immunostaining and analysis. A few hours after attachment to micropatterns hPSCs formed thin actin fiber assembly and cell membrane protrusions on the adhesive surface. Strong stress fibers (acto-myosin contraction) were observed along the non-adhesive cell edges in the manner reported for other cell types (Fig. 5).^{29,30} In contrast to hMSCs, hPSCs did not quickly form distinct vinculin stripes revealing focal adhesion sites on the vitronectin patterns (Fig. 5(G)) but rather dot-like focal complexes were observed (data not shown) which are evidence of cell spreading and migratory activity on adhesive substrate.³¹ However cell focal adhesions were clearly seen on micropatterned ECM after sustained culture (Figs. 5(C), (I)). The difference in cell attachment and spreading on micropatterns between hMSCs and hPSCs may reflect a less differentiated phenotype and an undeveloped cytoskeleton of the latter.^{32,33} Surprisingly, single hPSCs were able to attach on patterns in the presence of ROCK inhibitor (Figs. 5(D), (E) and (I)) as well as in its absence (Figs. 5(A)–(C), (F)–(H)). Single hPSCs have also consistently expressed pluripotency marker OCT4 (Figs. 5(A), (B), (D)–(F), (H)) when attached to vitronectin micropatterns. Further investigation is needed to determine whether control of the hPSC cytoskeleton through micropatterns induces changes in the expression of other pluripotency-associated transcription factors and affects hPSC gene expression profile.

Cell seeding density on these micropatterns needs to be optimized depending on cell type and size. Larger sizes of patterns can potentially result in incomplete and non-specific cell spreading. Higher plating densities of hPSCs (over 300,000 cells/chip) can result in up to 76% of patterns being filled but can also result in patterns being occupied by multiple cells (data not shown). Cells of hiPSC line BM9 (grown in a monolayer culture) more readily attached to vitronectin patterns in comparison with hESCs and furthermore hESC line HES3 (grown in colonies) demonstrated better performance compared to hESC line H9 (grown in a monolayer culture) on micropatterns.

The types of micropatterns described in this report have been previously applied to investigate mitotic spindle positioning and cell division in somatic cells^{10–12} with Kwon et al. having shown that adhesive micropatterns can control mitosis in aneuploid cancer cells.³⁴ The fibronectin patterns “O” and “Y” significantly increased formation of multipolar spindles in cells but in contrast, “H”-shaped patterns suppressed multipolar spindle formation and promoted bipolar cell division. This study revealed the possible role of extracellular microenvironment (specifically, adhesion sites) in the genomic stability of tumor cells and cancer progression.³⁴ hPSCs share many characteristics with cancer cells and can acquire chromosomal abnormalities and aneuploidy during cell culture.³⁵ Patient-specific hPSCs have been suggested for cell replacement therapies and tissue engineering applications and therefore the investigation of hPSC behavior and fate decision on adhesive constraints can provide particular benefits.

It has also been reported that the adhesive microenvironment can direct cell polarization and internal organization.¹ For instance the “crossbow” pattern induces asymmetric distribution of cell compartments and can potentially lead to asymmetric cell division (ACD).³⁰ This

pattern as well as a combination of the adhesive patterns “H” and “T” could potentially be used to study symmetric and asymmetric division of hPSCs.^{1, 30, 34}

ACD plays an important role in embryonic development, tissue homeostasis, and cancer. Tissue homeostasis is precisely controlled by microenvironmental cues.⁹ Any perturbations in this complex regulation, for example, in epithelial tissue may lead to the loss of tissue organization, cell polarity, and cell-cell contacts and may affect the ratio of symmetric to asymmetric divisions and lead to epithelial-to-mesenchymal transition (EMT) and malignant transformation of cells.¹³ Interestingly, hPSCs grown in colonies demonstrate epithelial tissue-like organization with distinct apical-basal polarity.³⁶ The disruption of this structure leads to EMT, which is the first step in hPSC differentiation.^{37–39}

It has been well established that a stem cell undergoing symmetric division will produce an equal daughter stem cell and in the case of asymmetric division a stem cell will produce a different progenitor cell which is only capable of giving rise to a more differentiated progeny and terminally differentiated tissue-specific cells.^{40, 41} Cell division axis and mitotic spindle orientation determine the future position of a daughter cell and therefore the ultimate cell fate. These processes have been well studied on model organisms such as *C. Elegans*, *Drosophila*, and *Xenopus*^{40–42} however little is known about ACD in hPSCs and about its role in hPSC fate decision.

It is generally assumed that hPSCs undergo symmetric divisions while self-renewing and asymmetric divisions during differentiation, however a recent paper has demonstrated that in self-renewal divisions of hESCs cultured on a MEF feeder layer the distribution of pSMAD1 proteins destined for degradation actually was not symmetric.⁴³ Mammalian polarity proteins LGN and NuMa are members of *Gai*/NuMa/LGN non-canonical G-protein signaling pathway and play important roles in mitotic cell spindle orientation and ACD.^{44, 45} Moreover, it has been reported that different distributions of LGN protein in human neural stem cells can depend on culture conditions supporting either self-renewal or differentiation.⁴⁵ Unfortunately the role of LGN and NuMa in hPSC symmetric or asymmetric division has not yet been investigated so little is known about the behavior of these proteins in hPSCs induced to divide symmetrically or asymmetrically on ECM micropatterns.

Our attempts to study single hPSCs entering metaphase stage on patterns or in a monolayer culture a few hours postseeding have not been successful. Synchronization of hPSCs in a monolayer culture⁴⁶ gave similar amount of cells (~ 30%) at metaphase stage as in routine cell culture in colonies on Day 3–4 after passaging. Cell passaging after synchronization was obviously damaging and thus we did not use the synchronization in further experiments. Furthermore, no cells at metaphase stage were observed a few hours from cell plating on patterns and after cell spreading including the day following passaging (data not shown). Prolonged cell culture on patterns or the sorting of cells at the same stage of the cell cycle may potentially solve this problem but further studies are required to define optimal conditions for the investigation of hPSC division on single cell patterns.

4. CONCLUSIONS

In this study we have described a convenient and simple method for the design of micropatterns for single cell studies. Particularly we have developed conditions for vitronectin binding to chemically modified PS which makes it possible to conduct research on hPSCs at a single cell level. The results of our studies demonstrate that single hPSC cytoskeleton and cell adhesion can be precisely controlled by ECM micropatterns. These results provide an opportunity for further investigation of hPSC division, cell–cell interactions, and to control stem cell fate. Studies of hPSCs at a subcellular level will undoubtedly advance the stem cell field and promote new applications in regenerative medicine.

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Fig. 1.
 (A) Single cell patterns on photomasks. (B) Vitronectin/Fibrinogen-AF488 patterns on polystyrene substrates (scale bar 25 μm).

Fig. 2.

(A)–(D) MEFs and (E), (F) hMSCs on single cell patterns (shown in left lower corners). F-actin was stained with Rhodamine Phalloidin-TRITC (red), nuclei were stained with DAPI (blue), additionally, hMSCs were immunostained for vimentin (green, (E)) and vinculin (green, (F)) (scale bar 25 μm).

Fig. 3. hESCs (H9) in a monolayer culture (P11S-passage 11 as single cells) express pluripotency markers OCT4 (A) and SOX2 (B), and demonstrate characteristic cytoskeleton organization (C) and (D) revealed by Rhodamine Phalloidin staining. Nuclei were stained with DAPI (blue). hESCs acquire a trisomy of chromosomes 12 and 20 if passaged without ROCK inhibitor (ROCKi) postseeding (E). The addition of ROCK inhibitor after cell passaging results in cells with normal and abnormal karyotypes (F) (scale bar 50 μ m).

Fig. 4. hiPSCs (BM9) in a monolayer culture (P19S-passagc 19 as single cells) express pluripotency markers OCT4 (A) and SOX2 (B) and demonstrate trisomy of chromosome 12 (C), if passaged without ROCK inhibitor postseeding (scale bar 50 μ m).

Fig. 5.

Single hPSCs on vitronectin patterns with or without addition of ROCK inhibitor (ROCKi) postseeding. BM9 hiPSCs (A)–(C), (F) and (I) are shown in blue, H9 hESCs (D), (E) and HES3 hESCs (G), (H) are shown in red. Corresponding patterns are shown in left lower corners (“cb”–crossbow). BM9 hiPSCs and H9 hESCs were grown in a monolayer culture with single-cell passaging and HES3 hESCs before plating on micropatterns were grown in colonics on Geltrex (to eliminate MEFs) and routinely passaged as clumps. BM9 hiPSC on pattern “O” (A) is shown on D0, BM9 hiPSCs on patterns “crossbow” (B), (C) and “T” (I) are shown on D1 after plating and BM9 hiPSC on pattern “H” (F) is shown on D2 (~48 hours after plating). For hiPSC on pattern “T” (I), ROCK inhibitor was added after cell plating and kept overnight. H9 hESCs on patterns “O” (D) and pattern “H” (E) are shown on Day1 (~24 hours after plating). ROCK inhibitor was added after cell plating and kept overnight. HES3 hESCs on patterns “Y” (G) and “T” (H) are shown on D0 (~4 hours after plating). (F-actin was stained with Rhodamine Phalloidin (red), nuclei were stained with DAPI (blue) or with antibodies specific to OCT4 (green), focal adhesions were immunostained with antibodies specific to vinculin (green) (scale bar 25 μ m).

Table I

Surface chemical composition of the control and UV/ozone-treated polymer samples (atomic%).

The type of plastic	UV/ozone treatment time (minutes)					
	00	1	1.5	2.5	4	7.5
<i>Polyvinylchloride (PVC)</i>						
PVC	C 90.65 O 9.35	C 87.84 O 12.16	–	C 85.92 O 14.08	C 81.55 O 18.45	–
Sample washed with EtOH after UV/ozone				C 89.18 O 10.82*		
<i>Celt culture polystyrene (PS)</i>						
Bacterial grade	C 99.63	–	–	C 80.93	–	–
Bacterial grade unmodified PS	O 0.37			O 19.07		
Tissue culture treated PS	C 87.43	–	–	–	–	–
Treated PS	O 12.57					
<i>Plastruct polystyrene (PS)</i>						
Unmodified PS	C 97.31 O 2.69	C 90.91 O 9.09	–	C 82.51 O 17.49	C 72.16 O 27.84	–
Sample washed with EtOH after UV/ozone					C 85.87 O 14.13*	
PS+EtOH wash before UV/ozone exposure	C 98.89 O 1.11	–	C 88.17 O 11.83	C 81.84 O 18.16	–	–
PS+EtOH wash before UV/ozone exposure through the glass mask	–	–	–	C 96.33 O 3.67	–	C 95.73 O 4.27